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METHODS AND COMPOSITIONS FOR MODULATING T LYMPHOCYTES

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METHODS AND COMPOSITIONS FOR MODULATING T LYMPHOCYTES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Patent Application Serial No. 60/442,792 (filed January 25, 2003), the disclosure of which is incorporated herein by reference in its entirety and for all purposes.

FIELD OF THE INVENTION

The present invention generally relates to novel modulators of IP3K kinases and therapeutic applications of such modulators. More particularly, the invention pertains to novel IP3K modulators that modulate T lymphocyte differentiation and function, and to methods of using such modulators to treat diseases and conditions mediated by abnormal T cell activities.

BACKGROUND OF THE INVENTION

In the development of the immune system, T lymphocytes are derived from precursor stem cells which enter the thymus to undergo differentiation and maturation. T lymphocyte differentiation normally occurs via a series of discrete developmental stages involving an initial primitive progenitor cell without lymphocyte specific cell surface markers ($CD34^+ CD3^- CD4^- CD8^-$), followed by acquisition of lymphocyte specific markers and loss of $CD34$ ($CD34^- CD3^+ CD4^+ CD8^+$), followed by differentiation into mature $CD3^+$ T cells expressing either $CD4$ or $CD8$ ($CD3^+ CD4^+ CD8^-$ or $CD3^+ CD4^- CD8^+$).

While normal T cells are an integral part of mammalian immune responses, in some instances it is desirable to inhibit undesirable immune responses such as undesirable proliferation of T cells. For instance, autoimmune diseases are characterized as an immune reaction against "self" antigens. Autoimmune diseases include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS). T cell responses have also been implicated in graft rejection and graft versus host disease (GVHD). Thus, treatment

directed to inhibition of T cell differentiation would be greatly desired to treat such undesired immune responses.

There is a need for new compounds and methods for inhibiting T cell immune responses and for treating the above-noted diseases and conditions. By providing novel methods and compositions for modulating T cell development and functions, the instant invention fulfills this and other needs.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides methods for identifying agents that modulate T lymphocyte development or function. The methods entail assaying a cellular activity of an inositol 1,4,5-trisphosphate 3-kinase (IP3K) or a fragment thereof in the presence of a test compound to identify an agent that modulates the cellular activity of the IP3K. In some methods, the IP3K employed is an IP3KB. In some methods, the test compound inhibits kinase activity of the IP3K. These methods can further comprise testing the identified agents for ability to modulate T cell differentiation. T cell differentiation to be tested can be development of CD4+ CD8+ T cells into CD4+ or CD8+ mature T cells.

In a related aspect, the invention provides for identifying agents that modulate T lymphocyte differentiation. These methods comprise (a) assaying a cellular activity of an inositol 1,4,5-trisphosphate 3-kinase (also termed IP3K or ITPK) or a fragment thereof in the presence of a test agent to identify one or more modulating agents that modulate the cellular activity of the IP3KB (also termed ITPKB), and (b) testing one or more of the modulating agents for ability to modulate T lymphocyte development or function.

In some of these methods, the test compound inhibits kinase activity of the IP3K. Some methods assay the kinase activity in catalyzing conversion of inositol 1,4,5-triphosphate (IP3) to inositol 1,3,4,5-tetrakisphosphate (IP4). In some methods, the IP3K employed is an IP3KB. The IP3KB employed can have the amino acid sequence of Accession No. CAB65055, Accession No. CAC40660, Accession No. NP_002212 or that of SEQ ID NO: 1, or that is substantially identical to any of these sequences. It can also be encoded by a polynucleotide having a nucleotide sequence that is shown in SEQ ID NO: 2, 3, or 4, or that is substantial identical to any of these sequences.

In some other methods, the modulating agents decrease cellular levels of the IP3K. The cells can be selected from the group consisting of thymus cells, CD4+ CD8+ T cells, CD4+ T cells, CD8+ T cells, and NK cells. In some of these methods, the modulating agents inhibit expression of a gene encoding the IP3K.

In another aspect, the invention provides methods for suppressing an undesired T lymphocyte response in a subject. Such methods comprise administering to the subject an effective amount of an agent that inhibits a cellular activity of an IP3K, thereby suppressing T lymphocyte responses in the subject. Some of these methods are directed to subjects that suffer from an autoimmune disease or graft rejection. Examples of autoimmune diseases that are amenable to these methods include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), or multiple sclerosis (MS).

In another related aspect, the invention provides methods for modulating T lymphocyte differentiation in a subject. The methods comprise (a) screening test compounds to identify a modulating agent that modulates a cellular activity of an IP3K, and (b) administering to the subject a pharmaceutical composition comprising an effective amount of the modulating agent; thereby modulating T lymphocyte differentiation in the subject.

In another aspect, the invention provides methods for treating a disease or disorder in a subject, e.g., inflammation, graft versus host disease, psoriasis, or allergy (asthma, rhinitis, COPD, and dermatitis). These methods entail screening test compounds to identify a modulating agent that modulates a cellular activity of an IP3K, and then administering to the subject a pharmaceutical composition comprising an effective amount of the modulating agent; thereby treating the disease or disorder in the subject.

A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows that numbers of peripheral blood CD4+ and CD8+ T cells, but not B cells, are drastically reduced in *Ms. T-less* mutant mice.

Figure 2 shows that that spleens from *Ms. T-less* mutant mice are devoid of CD4+ and CD8+ T cells.

Figure 3 shows that *Ms. T-less* mice exhibit a block of T cell development at the double positive (DP) stage.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Overview

The invention is predicated in part on the discovery by the present inventors that a gene not known to be involved in T or B cell development plays an important and essential role in T cell development and function. It was found that a mouse with a recessive mutant in the inositol 1,4,5-trisphosphate 3-kinase B (IP3KB or ITPKB) gene lacks any T cells in the peripheral blood and displays a moderate perturbation of transitional B cell development in the spleen (termed “*Ms. T-less* mice”). The IP3KB gene was mapped to a small portion of chromosome 1. There are no genes in this region which are known to be involved in T or B cell development.

As detailed in the Examples below, closer analysis of T cell development in the thymus revealed a complete block at the CD4+CD8+ double positive (DP) stage with no CD4+ or CD8+ single positive (SP) cells present. Expression analysis of activation markers and T cell receptor (TCR) associated genes suggests that the DP cells in *Ms. T-less* mice are not receiving or improperly translating differentiation signals originating from the TCR. However, there were no significant effects on calcium responses in TCR-stimulated CD4+ CD8+ T cells from mice with this mutant IP3KB gene. Instead, it was found that there is a specific defect in the activation of Erk, a critical mediator of positive selection. This indicates that the present inventors have identified a novel and important component of lymphocyte maturation, i.e., revealing IP3KB as a unique and novel link between the TCR and the Ras-MAP kinase pathway which is essential for T cell development.

In accordance with these discoveries, the present invention provides methods for screening novel agents that modulate T cell development and function. Test agents are examined for their ability to modulate a cellular activity (e.g., cellular level or kinase activity) of an inositol 1,4,5-trisphosphate 3-kinase. Various IP3K enzymes can be employed in the screening assays. For example, either IP3KA, IP3KB, or IP3KC from human, rat or mouse can be used to screen the modulators. In preferred embodiments, an

IP3KB is used. In some preferred embodiments, the modulators identified in the screening assays inhibit IP3KB kinase activity or reduce its cellular level.

The invention also provides methods for modulating T cell development and function in a subject (including human and animals such as other mammals). The methods entail administering to a subject an IP3K modulator. The IP3K modulator can be identified in accordance with the present invention. Alternatively, the IP3K modulators employed in the methods can be IP3K inhibitors known in the art. For example, purine-based IP3K inhibitors are described in Chang et al., *ChemBioChem* 3:897-901, 2002. In some preferred methods, IP3KB inhibitors are used to inhibit T cell development and function. By modulating T cell development or function, these IP3K modulators also find applications in treating various medical conditions where undesired immune responses mediated by T cells play a role. Accordingly, the invention also provides methods for treating conditions such as autoimmune diseases or other conditions with undesired T cell responses.

Transgenic or knockout animals (e.g., mice) that do not express endogenous IP3KB are also provided in the invention. The animals can either have a null allele in the IP3KB locus or a mutation in the IP3KB gene which results in no functional IP3KB protein being produced. For example, the *Ms. T-less* mice described in the Examples below have a premature STOP codon at amino acid position 199 within exon 2 of the IP3KB gene. This results in expression of a nonfunctional protein that lacks most of the IP3KB sequence. By blocking differentiation of progenitor T cells into mature CD4+ or CD8+ T cells, such animals provide useful tools to study T cell development and T cell immune responses.

The following sections provide guidance for making and using the compositions of the invention, and for carrying out the methods of the invention.

II. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention pertains. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY (2d ed. 1994); THE CAMBRIDGE DICTIONARY OF SCIENCE AND TECHNOLOGY (Walker ed., 1988); and Hale & Marham, THE HARPER COLLINS DICTIONARY

OF BIOLOGY (1991). In addition, the following definitions are provided to assist the reader in the practice of the invention.

The term "agent" or "test agent" includes any substance, molecule, element, compound, entity, or a combination thereof. It includes, but is not limited to, e.g., protein, polypeptide, small organic molecule, polysaccharide, polynucleotide, and the like. It can be a natural product, a synthetic compound, or a chemical compound, or a combination of two or more substances. Unless otherwise specified, the terms "agent", "substance", and "compound" can be used interchangeably.

The term "analog" is used herein to refer to a molecule that structurally resembles a reference molecule but which has been modified in a targeted and controlled manner, by replacing a specific substituent of the reference molecule with an alternate substituent. Compared to the reference molecule, an analog would be expected, by one skilled in the art, to exhibit the same, similar, or improved utility. Synthesis and screening of analogs, to identify variants of known compounds having improved traits (such as higher binding affinity for a target molecule) is an approach that is well known in pharmaceutical chemistry.

As used herein, "contacting" has its normal meaning and refers to combining two or more agents (e.g., polypeptides or small molecule compounds) or combining agents and cells (e.g., a polypeptide and a cell). Contacting can occur *in vitro*, e.g., combining two or more agents or combining a test agent and a cell or a cell lysate in a test tube or other container. Contacting can also occur *in a cell* or *in situ*, e.g., contacting two polypeptides in a cell by coexpression in the cell of recombinant polynucleotides encoding the two polypeptides, or in a cell lysate.

A "heterologous sequence" or a "heterologous nucleic acid," as used herein, is one that originates from a source foreign to the particular host cell, or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that, although being endogenous to the particular host cell, has been modified. Modification of the heterologous sequence can occur, e.g., by treating the DNA with a restriction enzyme to generate a DNA fragment that is capable of being operably linked to the promoter. Techniques such as site-directed mutagenesis are also useful for modifying a heterologous nucleic acid.

The term “homologous” when referring to proteins and/or protein sequences indicates that they are derived, naturally or artificially, from a common ancestral protein or protein sequence. Similarly, nucleic acids and/or nucleic acid sequences are homologous when they are derived, naturally or artificially, from a common ancestral nucleic acid or nucleic acid sequence. Homology is generally inferred from sequence similarity between two or more nucleic acids or proteins (or sequences thereof). The precise percentage of similarity between sequences that is useful in establishing homology varies with the nucleic acid and protein at issue, but as little as 25% sequence similarity is routinely used to establish homology. Higher levels of sequence similarity, e.g., 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more can also be used to establish homology. Methods for determining sequence similarity percentages (e.g., BLASTP and BLASTN using default parameters) are described herein or well known and readily available in the art.

A “host cell,” as used herein, refers to a prokaryotic or eukaryotic cell that contains heterologous DNA that has been introduced into the cell by any means, e.g., electroporation, calcium phosphate precipitation, microinjection, transformation, viral infection, and/or the like.

The terms “identical” or “sequence identity” in the context of two nucleic acid sequences or amino acid sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window. A “comparison window”, as used herein, refers to a segment of at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are aligned optimally. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2:482; by the alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443; by the search for similarity method of Pearson and Lipman (1988) *Proc. Nat. Acad. Sci. U.S.A.* 85:2444; by computerized implementations of these algorithms (including, but not limited to CLUSTAL in the PC/Gene program by Intelligentics, Mountain View, CA; and GAP, BESTFIT, BLAST, FASTA, or TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis., U.S.A.). The CLUSTAL program is well described by Higgins and Sharp (1988) *Gene*

73:237-244; Higgins and Sharp (1989) CABIOS 5:151-153; Corpet et al. (1988) Nucleic Acids Res. 16:10881-10890; Huang et al (1992) Computer Applications in the Biosciences 8:155-165; and Pearson et al. (1994) Methods in Molecular Biology 24:307-331. Alignment is also often performed by inspection and manual alignment. In one class of embodiments, the polypeptides herein are at least 70%, generally at least 75%, optionally at least 80%, 85%, 90%, 95% or 99% or more identical to a reference polypeptide, as measured by BLASTP (or CLUSTAL, or any other available alignment software) using default parameters. Similarly, nucleic acids can also be described with reference to a starting nucleic acid, e.g., they can be 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more identical to a reference nucleic acid, as measured by BLASTN (or CLUSTAL, or any other available alignment software) using default parameters.

The terms “substantially identical” nucleic acid or amino acid sequences means that a nucleic acid or amino acid sequence comprises a sequence that has at least 90% sequence identity or more, preferably at least 95%, more preferably at least 98% and most preferably at least 99%, compared to a reference sequence using the programs described above (preferably BLAST) using standard parameters. For example, the BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)). Percentage of sequence identity is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over

at least about 150 residues. In a most preferred embodiment, the sequences are substantially identical over the entire length of the coding regions.

The term “isolated” means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring nucleic acid, polypeptide, or cell present in a living animal is not isolated, but the same polynucleotide, polypeptide, or cell separated from some or all of the coexisting materials in the natural system, is isolated, even if subsequently reintroduced into the natural system. Such nucleic acids can be part of a vector and/or such nucleic acids or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The terms “nucleic acid,” “DNA sequence” or “polynucleotide” refer to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in manner similar to naturally occurring nucleotides. A “polynucleotide sequence” is a nucleic acid (which is a polymer of nucleotides (A,C,T,U,G, etc. or naturally occurring or artificial nucleotide analogues) or a character string representing a nucleic acid, depending on context. Either the given nucleic acid or the complementary nucleic acid can be determined from any specified polynucleotide sequence.

The term “modulate” with respect to cellular activities of an IP3K refers to a change in the cellular level or other biological activities (e.g., kinase activity) of the IP3K enzyme. Modulation of IP3K activities can be up-regulation (i.e., activation or stimulation) or down-regulation (i.e. inhibition or suppression). For example, modulation may cause a change in cellular level of IP3K, enzymatic modification (e.g., phosphorylation) of IP3K, binding characteristics (e.g., binding to a substrate or ATP), or any other biological, functional, or immunological properties of IP3K proteins. The change in activity can arise from, for example, an increase or decrease in expression of the IP3K gene, the stability of mRNA that encodes the IP3K protein, translation efficiency, or from a change in other bioactivities of the IP3K enzymes (e.g., its kinase activity). The mode of action of an IP3K modulator can be direct, e.g., through binding to the IP3K protein or to a gene encoding the IP3K protein. The change can also be indirect, e.g., through binding to and/or modifying (e.g., enzymatically) another molecule which otherwise modulates IP3K.

The term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

The term "operably linked" refers to a functional relationship between two or more polynucleotide (e.g., DNA) segments. Typically, it refers to the functional relationship of a transcriptional regulatory sequence to a transcribed sequence. For example, an IP3K promoter or enhancer sequence, is operably linked to a coding sequence if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell or other expression system. Generally, promoter transcriptional regulatory sequences that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are *cis*-acting. However, some transcriptional regulatory sequences, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance. A polylinker provides a convenient location for inserting coding sequences so the genes are operably linked to the IP3K promoter. Polylinkers are polynucleotide sequences that comprise a series of three or more closely spaced restriction endonuclease recognition sequences.

The term "polypeptide" is used interchangeably herein with the terms "polypeptides" and "protein(s)", and refers to a polymer of amino acid residues, e.g., as typically found in proteins in nature. A "mature protein" is a protein which is full-length and which, optionally, includes glycosylation or other modifications typical for the protein in a given cell membrane.

The promoter region of a gene includes the transcription regulatory elements that typically lie 5' to a structural gene. If a gene is to be activated, proteins known as transcription factors attach to the promoter region of the gene. This assembly resembles an "on switch" by enabling an enzyme to transcribe a second genetic segment from DNA into RNA. In most cases the resulting RNA molecule serves as a template for synthesis of a specific protein; sometimes RNA itself is the final product. The promoter region may be a normal cellular promoter or an oncogene.

Transcription refers to the process involving the interaction of an RNA polymerase with a gene, which directs the expression as RNA of the structural information present in the coding sequences of the gene. The process includes, but is not limited to the following steps: (1) transcription initiation, (2) transcript elongation, (3) transcript splicing, (4) transcript capping, (5) transcript termination, (6) transcript polyadenylation, (7) nuclear export of the transcript, (8) transcript editing, and (9) stabilizing the transcript.

A transcription regulatory element or sequence include, but is not limited to, a promoter sequence (e.g., the TATA box), an enhancer element, a signal sequence, or an array of transcription factor binding sites. It controls or regulates transcription of a gene operably linked to it.

A "variant" of a molecule such as an IP3K is meant to refer to a molecule substantially similar in structure and biological activity to either the entire molecule, or to a fragment thereof. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the sequence of amino acid residues is not identical.

III. Inositol 1,4,5-trisphosphate 3-kinases (IP3Ks) Used in Screening

Inositol 1,4,5-trisphosphate 3-kinases (IP3Ks) are enzymes which catalyze the conversion of inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃, or IP₃) to inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄ or IP4). IP3 and IP4 are potential modulators of calcium homoeostasis. There are three different isoforms of IP3K, IP3KA, IP3KB, and IP3KC. In the present invention, novel modulators of IP3K are identified by screening test agents for ability to modulate a cellular activity of an IP3K. The cellular activities to be monitored in the screening assays can be an enzymatic activity of the IP3K or its cellular level. Modulation of the cellular activity can be an up-regulation (to increase or stimulate) or a down-regulation (to decrease or inhibit). In preferred embodiments, test agents are screened for ability to inhibit the IP3K activity in catalyzing the conversion of IP3 to IP4.

Various IP3Ks can be employed in screening the IP3K modulators of the present invention. Preferably, an IP3KB molecule is used. In some methods, an IP3KB polypeptide having an amino acid sequence of Accession No. CAB65055, Accession No.

CAC40660, Accession No. NP_002212 or SEQ ID NO: 1, or a substantial identical sequence, is employed in the screening assay. In some methods, an IP3K polypeptide that is encoded by a polynucleotide having the sequence of SEQ ID NO: 2, 3, or 4, or a substantial identical sequence, is used. SEQ ID NOS: 2, 3, and 4 respectively encode IP3KB from human, rat, and mouse.

Other than IP3K sequences disclosed herein, various IP3K sequences that have been described in the art can also be employed in the screening assays of the present invention. For example, different isoforms of IP3K have been identified in a number of species, see, e.g., Takazawa et al., Rat brain inositol 1,4,5-trisphosphate 3-kinase. Ca 2+ -sensitivity, purification and antibody production. *Biochem. J.* 268, 213-217, 1990; Thomas et al., Isolation and sequence of a full length cDNA encoding a novel rat inositol 1,4,5-trisphosphate 3-kinase. *Biochim. Biophys. Acta* 1220, 219-222, 1994; Takazawa et al., Molecular cloning and expression of a human brain inositol 1,4,5-trisphosphate 3-kinase. *Biochem. Biophys. Res. Commun.* 174, 529-535, 1991; Takazawa et al., Molecular cloning and expression of a new putative inositol 1,4,5-trisphosphate 3-kinase isoenzyme. *Biochem. J.* 278, 883-886, 1991; Vanweyenberg et al., Tissue- and cell-specific expression of Ins(1,4,5)P 3 3-kinase isoenzymes. *Biochem. J.* 306, 429-435, 1995. Dewaste et al., Cloning and expression of a cDNA encoding human inositol 1,4,5-trisphosphate 3-kinase C. *Biochem. J.* 352: 343-351, 2000; Choi et al., Molecular cloning and expression of a complementary DNA for inositol 1,4,5-trisphosphate 3-kinase. *Science* 248: 64-66, 1990; and Mailleux et al., Astrocytic localization of the messenger RNA encoding the isoenzyme B of inositol (1,4,5) 3-kinase in the human brain. *Neurosci. Lett.* 148: 177-180, 1992. Yeast and *C. elegans* IP3Ks have also been described in the art. See, e.g., GenBank Accession Numbers P91166 and NP-010458 and Dewaste et al., *Biochem. J.* 352: 343-351, 2000. Additional IP3K sequences or fragments from various species have also been described in the art, e.g., amino acid sequences with Accession Numbers P27987, P23677, P17105, Q91XW1, CG1630, O45049, Q9Y475, Q9YH86, and Q963D4 (EC 2.7.1.127). Other polynucleotide sequences encoding human, mouse, or rat IP3Ks include, but are not limited to, accession numbers Y18024, NM002221, BC015009, AJ242780, AJ242781, AK0533759, AK050506, and NM019312. Any of these IP3K sequences can be used to screen test agents for modulators in the present invention.

In addition to an intact IP3K molecule or a polynucleotide encoding the intact IP3K molecule, an IP3K fragment, analog, or a functional derivative can also be used. The IP3K fragments that can be employed in these assays usually retain one or more of the biological activities of the IP3K molecule (typically, its kinase activity). For example, the three isoforms of IP3Ks share a conserved catalytic domain of about 275 amino acids (Dewaste et al., *Biochem. J.* 352: 343-351, 2000). ATP binding site and IP3 binding site are often also preserved in such fragments or analogs. Fusion proteins containing such fragments or analogs can also be used for the screening of test agents. Similarly, functional derivatives of IP3Ks usually have amino acid deletions and/or insertions and/or substitutions while maintaining one or more of the bioactivities. As noted above, IP3Ks from the different species have already been sequenced and well characterized. Therefore, their fragments, analogs, derivatives, or fusion proteins can be easily obtained using methods well known in the art. For example, a functional derivative of an IP3K can be prepared from a naturally occurring or recombinantly expressed protein by proteolytic cleavage followed by conventional purification procedures known to those skilled in the art. Alternatively, the functional derivative can be produced by recombinant DNA technology by expressing only fragments of an IP3K that retains its kinase activity.

IV. Test Agents

Test agents that can be screened with methods of the present invention include polypeptides, beta-turn mimetics, polysaccharides, phospholipids, hormones, prostaglandins, steroids, aromatic compounds, heterocyclic compounds, benzodiazepines, oligomeric N-substituted glycines, oligocarbamates, polypeptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Some test agents are synthetic molecules, and others natural molecules.

Test agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. Combinatorial libraries can be produced for many types of compound that can be synthesized in a step-by-step fashion. Large combinatorial libraries of compounds can be constructed by the encoded synthetic libraries (ESL) method described in WO 95/12608, WO 93/06121, WO 94/08051, WO 95/35503 and WO 95/30642. Peptide libraries can also be generated by phage display methods (see, e.g., Devlin, WO 91/18980).

Libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts can be obtained from commercial sources or collected in the field. Known pharmacological agents can be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

Combinatorial libraries of peptides or other compounds can be fully randomized, with no sequence preferences or constants at any position. Alternatively, the library can be biased, i.e., some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in some cases, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, or to purines.

The test agents can be naturally occurring proteins or their fragments. Such test agents can be obtained from a natural source, e.g., a cell or tissue lysate. Libraries of polypeptide agents can also be prepared, e.g., from a cDNA library commercially available or generated with routine methods. The test agents can also be peptides, e.g., peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides can be digests of naturally occurring proteins, random peptides, or “biased” random peptides. In some methods, the test agents are polypeptides or proteins.

The test agents can also be nucleic acids. Nucleic acid test agents can be naturally occurring nucleic acids, random nucleic acids, or “biased” random nucleic acids. For example, digests of prokaryotic or eukaryotic genomes can be similarly used as described above for proteins.

In some preferred methods, the test agents are small molecules (e.g., molecules with a molecular weight of not more than about 1,000). Preferably, high throughput assays are adapted and used to screen for such small molecules. In some methods, combinatorial libraries of small molecule test agents as described above can be readily employed to screen for small molecule modulators of IP3Ks. A number of assays are available for such screening, e.g., as described in Schultz (1998) *Bioorg Med Chem Lett* 8:2409-2414; Weller (1997) *Mol Divers.* 3:61-70; Fernandes (1998) *Curr Opin Chem Biol* 2:597-603; and Sittampalam (1997) *Curr Opin Chem Biol* 1:384-91.

Libraries of test agents to be screened with the claimed methods can also be generated based on structural studies of the IP3K polypeptides, their fragments or analogs. Such structural studies allow the identification of test agents that are more likely to bind to the IP3K polypeptides. The three-dimensional structure of an IP3K polypeptide can be studied in a number of ways, e.g., crystal structure and molecular modeling. Methods of studying protein structures using x-ray crystallography are well known in the literature. See Physical Bio-chemistry, Van Holde, K. E. (Prentice-Hall, New Jersey 1971), pp. 221-239, and Physical Chemistry with Applications to the Life Sciences, D. Eisenberg & D. C. Crothers (Benjamin Cummings, Menlo Park 1979). Computer modeling of IP3K polypeptides' structures provides another means for designing test agents for screening IP3K modulators. Methods of molecular modeling have been described in the literature, e.g., U.S. Patent No. 5,612,894 entitled "System and method for molecular modeling utilizing a sensitivity factor", and U.S. Patent No. 5,583,973 entitled "Molecular modeling method and system". In addition, protein structures can also be determined by neutron diffraction and nuclear magnetic resonance (NMR). See, e.g., Physical Chemistry, 4th Ed. Moore, W. J. (Prentice-Hall, New Jersey 1972), and NMR of Proteins and Nucleic Acids, K. Wuthrich (Wiley-Interscience, New York 1986).

Modulators of the present invention also include antibodies that specifically bind to an IP3K polypeptide. Such antibodies can be monoclonal or polyclonal. Such antibodies can be generated using methods well known in the art. For example, the production of non-human monoclonal antibodies, e.g., murine or rat, can be accomplished by, for example, immunizing the animal with an IP3K polypeptide or its fragment (See Harlow & Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor New York). Such an immunogen can be obtained from a natural source, by peptides synthesis or by recombinant expression.

Humanized forms of mouse antibodies can be generated by linking the CDR regions of non-human antibodies to human constant regions by recombinant DNA techniques. See Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989) and WO 90/07861. Human antibodies can be obtained using phage-display methods. See, e.g., Dower et al., WO 91/17271; McCafferty et al., WO 92/01047. In these methods, libraries of phage are produced in which members display different antibodies on their outer surfaces. Antibodies are usually displayed as Fv or Fab fragments. Phage displaying antibodies with a

desired specificity are selected by affinity enrichment to an IP3K polypeptide of the present invention.

Human antibodies against an IP3K polypeptide can also be produced from non-human transgenic mammals having transgenes encoding at least a segment of the human immunoglobulin locus and an inactivated endogenous immunoglobulin locus. See, e.g., Lonberg et al., WO93/12227 (1993); Kucherlapati, WO 91/10741 (1991). Human antibodies can be selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a particular mouse antibody. Such antibodies are particularly likely to share the useful functional properties of the mouse antibodies. Human polyclonal antibodies can also be provided in the form of serum from humans immunized with an immunogenic agent. Optionally, such polyclonal antibodies can be concentrated by affinity purification using an IP3K polypeptide or its fragment.

V. Screen Test Agents for IP3K Modulators

Employing an IP3K polypeptide described above, the present invention provides methods for screening agents or compounds that modulate cellular activities (e.g., kinase activity or cellular level) of IP3Ks. In some preferred embodiments, test agents are screened for ability to modulate (e.g., inhibit) IP3K activity in catalyzing IP3 conversion to IP4. A variety of routinely practiced assays can be used to identify test agents that modulate kinase activity of an IP3K. For example, inhibitors of an IP3K can be identified in a soluble assay format as described in Chang et al., *Chembiochem* 3:897-901, 2002. Dewaste et al. (*Biochem. J.* 352: 343-351, 2000) disclosed a method wherein IP3K kinase activity was determined in bacteria and in transfected cells under basal conditions. Woodring et al. (*J. Biol. Chem.* 272: 30447-54, 1997) also described IP3K activity assays. These assays often use labeled or unlabeled IP3 and ATP in the presence of an IP3K kinase and a test agent. High performance liquid chromatography (HPLC) can be used to quantitated IP4 in the reaction product. Alternatively, conversion of IP3 to IP4 can be determined by measuring radioactivity incorporated into IP4 when IP3 used in the reaction is radiolabeled (Chang et al., *Chembiochem* 3: 897-901, 2002). IP3K activity can also be monitored by the kinase glow assay. This assay measures the disappearance of labeled ATP once it has been used to phosphorylate the substrate. With this assay format, the requirement for detecting the formation of IP4 from IP3 can be bypassed. The kinase glow assay is well known and

routinely practiced in the art, e.g., as described in Somberg et al., Promega Notes, 83: 14-17, 2003. Any of these assays can be readily adopted in the present invention to screen for modulators of IP3K kinase activity.

In some methods, test agents can be first screened for their ability to bind to an IP3K polypeptide. Compounds thus identified can be further subject to assay for ability to modulate (e.g., to inhibit) IP3K kinase activity as described above. Binding of test agents to an IP3K polypeptide can be assayed by a number of methods including, e.g., labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.), and the like. See, e.g., U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168; and also Bevan et al., Trends in Biotechnology 13:115-122, 1995; Ecker et al., Bio/Technology 13:351-360, 1995; and Hodgson, Bio/Technology 10:973-980, 1992. The test agent can be identified by detecting a direct binding to the IP3K polypeptide, e.g., co-immunoprecipitation with the IP3K polypeptide by an antibody directed to the IP3K polypeptide. The test agent can also be identified by detecting a signal that indicates that the agent binds to the IP3K polypeptide, e.g., fluorescence quenching.

Competition assays provide a suitable format for identifying test agents that specifically bind to an IP3K polypeptide. In such formats, test agents are screened in competition with a compound already known to bind to the IP3K polypeptide. The known binding compound can be a synthetic compound. It can also be an antibody, which specifically recognizes the IP3K polypeptide, e.g., a monoclonal antibody directed against the IP3K polypeptide. If the test agent inhibits binding of the compound known to bind the IP3K polypeptide, then the test agent also binds the IP3K polypeptide.

Numerous types of competitive binding assays are known, for example: solid phase direct or indirect radioimmunoassay (RIA), solid phase direct or indirect enzyme immunoassay (EIA), sandwich competition assay (see Stahli et al., Methods in Enzymology 9:242-253 (1983)); solid phase direct biotin-avidin EIA (see Kirkland et al., J. Immunol. 137:3614-3619 (1986)); solid phase direct labeled assay, solid phase direct labeled sandwich assay (see Harlow and Lane, "Antibodies, A Laboratory Manual," Cold Spring Harbor Press (1988)); solid phase direct label RIA using ¹²⁵I label (see Morel et al., Mol. Immunol. 25(1):7-15 (1988)); solid phase direct biotin-avidin EIA (Cheung et al., Virology 176:546-552 (1990)); and direct labeled RIA (Moldenhauer et al., Scand. J. Immunol. 32:77-82

(1990)). Typically, such an assay involves the use of purified polypeptide bound to a solid surface or cells bearing either of these, an unlabelled test agent and a labeled reference compound. Competitive inhibition is measured by determining the amount of label bound to the solid surface or cells in the presence of the test agent. Usually the test agent is present in excess. Modulating agents identified by competition assay include agents binding to the same epitope as the reference compound and agents binding to an adjacent epitope sufficiently proximal to the epitope bound by the reference compound for steric hindrance to occur. Usually, when a competing agent is present in excess, it will inhibit specific binding of a reference compound to a common target polypeptide by at least 50 or 75%.

The screening assays can be either in insoluble or soluble formats. One example of the insoluble assays is to immobilize an IP3K polypeptide or its fragments onto a solid phase matrix. The solid phase matrix is then put in contact with test agents, for an interval sufficient to allow the test agents to bind. Following washing away any unbound material from the solid phase matrix, the presence of the agent bound to the solid phase allows identification of the agent. The methods can further include the step of eluting the bound agent from the solid phase matrix, thereby isolating the agent. Alternatively, other than immobilizing the IP3K polypeptide, the test agents are bound to the solid matrix and the IP3K polypeptide molecule is then added.

Soluble assays include some of the combinatorial libraries screening methods. Under the soluble assay formats, neither the test agents nor the IP3K polypeptide are bound to a solid support. Binding of an IP3K polypeptide or fragment thereof to a test agent can be determined by, e.g., changes in fluorescence of either the IP3K polypeptide or the test agents, or both. Fluorescence may be intrinsic or conferred by labeling either component with a fluorophor.

In some binding assays, either the IP3K polypeptide, the test agent, or a third molecule (e.g., an antibody against the IP3K polypeptide) can be provided as labeled entities, i.e., covalently attached or linked to a detectable label or group, or cross-linkable group, to facilitate identification, detection and quantification of the polypeptide in a given situation. These detectable groups can comprise a detectable polypeptide group, e.g., an assayable enzyme or antibody epitope. Alternatively, the detectable group can be selected from a variety of other detectable groups or labels, such as radiolabels (e.g., ^{125}I , ^{32}P , ^{35}S) or

a chemiluminescent or fluorescent group. Similarly, the detectable group can be a substrate, cofactor, inhibitor or affinity ligand.

In some other methods, test agents are assayed for activity to modulate cellular levels of the IP3K polypeptide, e.g., transcription or translation. The test agent can also be assayed for activities in modulating expression level or stability of the IP3K polypeptide, e.g., post-translational modification or proteolysis. Various biochemical and molecular biology techniques well known in the art can be employed to practice the present invention. Such techniques are described in, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, N.Y., Second (1989) and Third (2000) Editions; and Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York (1987-1999). In some embodiments, endogenous levels of an IP3KB can be directly monitored in cells normally expressing this enzyme (e.g., thymus cells). In some embodiments, expression or cellular level of an IP3K can be examined in an expression system using cloned cDNA or genomic sequence encoding the IP3K.

Alternatively, modulation of expression of an IP3K gene can be examined in a cell-based system by transient or stable transfection of an expression vector into cultured cell lines. Assay vectors bearing transcription regulatory sequence (e.g., promoter) of an IP3K gene operably linked to reporter genes can be transfected into any mammalian host cell line for assays of promoter activity. Constructs containing an IP3K gene (or a transcription regulatory element of an IP3K gene) operably linked to a reporter gene can be prepared using only routinely practiced techniques and methods of molecular biology (see, e.g., Sambrook et al. and Ausubel et al., *supra*). General methods of cell culture, transfection, and reporter gene assay have been described in the art, e.g., Ausubel, *supra*; and Transfection Guide, Promega Corporation, Madison, WI (1998). Any readily transfectable mammalian cell line may be used to assay IP3K promoter function, e.g., HCT116, HEK 293, MCF-7, and HepG2 are all suitable cell lines.

When inserted into the appropriate host cell, the transcription regulatory elements in the expression vector induces transcription of the reporter gene by host RNA polymerases. Reporter genes typically encode polypeptides with an easily assayed enzymatic activity that is naturally absent from the host cell. Typical reporter polypeptides for eukaryotic promoters include, e.g., chloramphenicol acetyltransferase (CAT), firefly or

Renilla luciferase, beta-galactosidase, beta-glucuronidase, alkaline phosphatase, and green fluorescent protein (GFP).

VI. Modulation of T Cell Differentiation and Function

The present invention provides compositions and methods for modulating T cell development and functions. As a consequence of the connection between the IP3K and T cell development, modulation of cellular levels or kinase activity of IP3Ks (e.g., IP3KB) can lead to modulation of T cell function and immune response mediated by T cells. To identify such modulators of T cells, an IP3K modulator described above can be further examined to confirm its ability to modulate T cell differentiation.

As noted above, T lymphocyte differentiation proceeds through a series of discrete developmental stages. The progenitor cells (CD4⁻ CD8⁻) first differentiate into double positive (DP) T cells (CD4⁺ CD8⁺ cells). The DP T cells then further develop into one of the mature, single positive (SP) T cells, CD4⁺ CD8⁻ or CD4⁻ CD8⁺. T cell progenitor cells as used herein therefore include pluripotent cells which are capable of self-renewal and differentiation into all myeloid and lymphoid cell lineages, including T cells.

Unless otherwise specified, “T cell differentiation” is used interchangeably herein with “T cell development” or “T cell maturation.” These terms encompass the various stages of the process in which CD34+ progenitor cells develop into mature SP CD4+ or CD8+ T cells. Accordingly, activity of an IP3K modulator on T cell differentiation can be examined at any of these development stages. Modulation of T cell development can be examined using progenitor T cells at these various stages. In some embodiments, T cell development being modulated specifically refers to the development stage during which the DP (CD4+ CD8+) T cells develop into SP (CD4+ or CD8+) T cells.

T cell progenitor cells may be isolated from sources including bone marrow, umbilical cord blood or peripheral blood mobilized stem cells. Peripheral blood mobilized stem cells are obtained from the peripheral blood of subjects who have been treated with chemotherapeutic agents and/or cytokines to increase hematopoietic progenitor cells circulating in peripheral blood. The preferred hematopoietic T cell progenitor cells are those derived from humans. Progenitor cells at various stages of differentiation may be used in the present invention.

In some methods of the invention, modulation of T cell development by an IP3K modulating agent can be examined in vitro using thymic stromal cells derived from the disaggregation of a piece of thymus tissue. As described in, e.g., US Patent No. 5,677,139, this assay system is capable of supporting in vitro T cell growth and differentiation. Thymic stromal cells provide the supporting microenvironment in the intact thymus for the differentiation of T cell progenitor cells to mature T cells. The microenvironment includes soluble and cell surface factors expressed by the various cell types which comprise the thymic stroma.

Thymic stroma cells may be obtained from the thymus of a mammal (e.g., mice) or of a non-human primate at any time after the organ has developed to a stage at which it can support the differentiation of T cells. In primates, this stage of thymic development is achieved during the second trimester. At this stage of development the thymus can produce peptide hormones such as thymulin, thymosin, and thymopoietin, as well as other factors required to provide the proper microenvironment for T cell differentiation. It is preferred that the stromal cells are derived from a non-human primate thymus during the third trimester of gestation or from a thymus of a non-human primate neonate. During the mid to late third trimester, the thymus stromal microenvironment is fully capable of inducing the differentiation of T cell progenitor cells to mature T cells. The non-human primate stromal cells can be derived from any non-human primate. Examples include monkey, chimpanzee, and baboon.

In some other embodiments, other than using an in vitro system such as thymic stroma cells, modulating activity on T cell development is examined using an animal harboring an IP3K (e.g., IP3KB). The animal can endogenously express an IP3K (e.g., mice expressing mouse IP3KB). Alternatively, a transgenic mouse containing human IP3KB gene can be employed to study in vivo activity of a test agent or a pre-screened IP3K modulator on T cell development. Typically, thymi from transgenic mice administered with the IP3K modulating compound can be analyzed at various differentiation stages. For example, they can be analyzed by flow cytometry using antibodies against the different antigen markers of the T cells (e.g., antibodies against CD4 and CD8).

Transgenic animals (e.g., transgenic mice) harboring a heterologous IP3K gene (e.g., the human IP3KB gene) can be generated according to methods well known in the art. For example, techniques routinely used to create and screen for transgenic animals

have been described in, e.g., see Bijvoet (1998) *Hum. Mol. Genet.* 7:53-62; Moreadith (1997) *J. Mol. Med.* 75:208-216; Tojo (1995) *Cytotechnology* 19:161-165; Mudgett (1995) *Methods Mol. Biol.* 48:167-184; Longo (1997) *Transgenic Res.* 6:321-328; U.S. Patents Nos. 5,616,491 (Mak, et al.); 5,464,764; 5,631,153; 5,487,992; 5,627,059; 5,272,071; and WO 91/09955, WO 93/09222, WO 96/29411, WO 95/31560, and WO 91/12650.

In some embodiments, an IP3K gene linked to a reporter gene is injected into the embryo of a developing animal (typically a mouse) to generate a transgenic animal. Once integration of the transgene has been verified, tissues of the animal (e.g., lymphoid tissues) are then assayed for expression of the transgene. For example, where the IP3K gene is linked to a reporter gene, tissues of the transgenic animal may be assayed either for reporter gene RNA or for the enzymatic activity of the reporter polypeptide.

VII. Therapeutic Applications

The invention provides therapeutic compositions and methods for preventing or treating diseases and conditions due to abnormal T cell development or functions. For example, subjects with diseases or disorders such as inflammation, graft versus host disease, psoriasis, or allergy (asthma, rhinitis, COPD, and dermatitis) are all amenable to treatment with methods and compositions of the present invention. Specific and selective inhibition of an IP3K kinase (e.g., IP3KB) in T cells can inhibit T cell maturation, activation and function, resulting in profound immunosuppression. For example, IP3KB specifically accumulates only in the brain and in T cells or lymphoid tissues containing high numbers of T cells. Therefore, selective IP3KB inhibitors could prove highly tissue and even cell type specific. This will reduce the likelihood of adverse side reactions and general toxicity. As a result, therapeutic compositions comprising selective IP3KB-inhibitors of the present invention are advantageous over currently used immunosuppression drugs such as cyclosporine. The latter has severe side effects due to its pleiotrophic action.

Thus, the IP3K modulators of the present invention (e.g., a specific and selective IP3KB inhibitor) provide novel and less toxic immunosuppressants than those currently in clinical use. They are useful in the treatment of various medical conditions. In addition, other than the novel IP3K modulators of the present invention, IP3K inhibitors that are known in the art can also be employed in the therapeutic methods of the present invention (e.g., purine-based IP3K inhibitors as described in Chang et al., *Chembiochem*

3:897-901, 2002). Examples of the medical conditions include inflammatory responses in transplant rejection, autoimmune disease (e.g. systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis), graft rejection and graft versus host disease, psoriasis, allergy (asthma, rhinitis, COPD, dermatitis), and others. In addition to treating these diseases or conditions, IP3K modulators of the present invention (e.g., IP3KB inhibitors) are also useful for preventing or modulating the development of such diseases or disorders in a subject (including human and animals such as other mammals) suspected of being, or known to be, prone to such diseases or disorders. In some applications, an IP3K inhibitor of the present invention can also be administered to a subject along with known immunosuppressive drugs such as cyclosporin.

The IP3K modulators of the present invention can be directly administered under sterile conditions to the subject to be treated. The modulators can be administered alone or as the active ingredient of a pharmaceutical composition. The therapeutic composition of the present invention can also be combined with or used in association with other therapeutic agents.

Pharmaceutical compositions of the present invention typically comprise at least one active ingredient together with one or more acceptable carriers thereof. Pharmaceutically carriers enhance or stabilize the composition, or to facilitate preparation of the composition. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered (e.g., nucleic acid, protein, modulatory compounds or transduced cell), as well as by the particular method used to administer the composition. They should also be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the subject. This carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral, sublingual, rectal, nasal, or parenteral. For example, the IP3K modulator can be complexed with carrier proteins such as ovalbumin or serum albumin prior to their administration in order to enhance stability or pharmacological properties.

There are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (see, e.g., *Remington: The Science and Practice of Pharmacy*, Mack Publishing Co., 20th ed., 2000). Without limitation, they include syrup, water, isotonic saline solution, 5% dextrose in water or buffered sodium or ammonium acetate solution, oils, glycerin, alcohols, flavoring agents, preservatives, coloring agents

starches, sugars, diluents, granulating agents, lubricants, and binders, among others. The carrier may also include a sustained release material such as glyceryl monostearate or glyceryl distearate, alone or with a wax.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100% by weight. Therapeutic formulations are prepared by any methods well known in the art of pharmacy. See, e.g., Gilman et al., eds., Goodman and Gilman's: The Pharmacological Bases of Therapeutics , 8th ed., Pergamon Press, 1990; Remington: The Science and Practice of Pharmacy, Mack Publishing Co., 20th ed., 2000; Avis et al., eds., Pharmaceutical Dosage Forms: Parenteral Medications, published by Marcel Dekker, Inc., N.Y., 1993; Lieberman et al., eds., Pharmaceutical Dosage Forms: Tablets, published by Marcel Dekker, Inc., N.Y., 1990; and Lieberman et al., eds., Pharmaceutical Dosage Forms: Disperse Systems, published by Marcel Dekker, Inc., N.Y., 1990.

The therapeutic formulations can be delivered by any effective means which could be used for treatment. Depending on the specific IP3K modulators to be administered, the suitable means include oral, rectal, vaginal, nasal, pulmonary administration, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) infusion into the bloodstream. They can also be administered in eye drops or topical skin application.

For parenteral administration, IP3K modulators of the present invention may be formulated in a variety of ways. Aqueous solutions of the modulators may be encapsulated in polymeric beads, liposomes, nanoparticles or other injectable depot formulations known to those of skill in the art. The nucleic acids may also be encapsulated in a viral coat.

Additionally, the compounds of the present invention may also be administered encapsulated in liposomes. The compositions, depending upon its solubility, may be present both in the aqueous layer and in the lipidic layer, or in what is generally termed a liposomal suspension. The hydrophobic layer, generally but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surfactants such a diacetylphosphate, stearylamine, or phosphatidic acid, and/or other materials of a hydrophobic nature.

The compositions may be supplemented by active pharmaceutical ingredients, where desired. Optional antibacterial, antiseptic, and antioxidant agents may also be present in the compositions where they will perform their ordinary functions.

The therapeutic formulations can conveniently be presented in unit dosage form and administered in a suitable therapeutic dose. A suitable therapeutic dose can be determined by any of the well known methods such as clinical studies on mammalian species to determine maximum tolerable dose and on normal human subjects to determine safe dosage. Except under certain circumstances when higher dosages may be required, the preferred dosage of an IP3K modulator usually lies within the range of from about 0.001 to about 1000 mg, more usually from about 0.01 to about 500 mg per day.

The preferred dosage and mode of administration of an IP3K modulator can vary for different subjects, depending upon factors that can be individually reviewed by the treating physician, such as the condition or conditions to be treated, the choice of composition to be administered, including the particular IP3K modulator, the age, weight, and response of the individual subject, the severity of the subject's symptoms, and the chosen route of administration. As a general rule, the quantity of an IP3K modulator administered is the smallest dosage which effectively and reliably prevents or minimizes the conditions of the subjects. Therefore, the above dosage ranges are intended to provide general guidance and support for the teachings herein, but are not intended to limit the scope of the invention.

In some applications, a first IP3K modulator is used in combination with a second IP3K modulator in order to modulate T cell development and function to a more extensive degree than cannot be achieved when one IP3K modulator is used individually.

EXAMPLES

The following examples are offered to illustrate, but not to limit the present invention.

Example 1 Generation of *Ms. T-less* Mice

This Example describes identification of mice that harbor N-ethyl-N-nitrosourea (ENU) induced homozygous recessive mutations in the IP3KB gene. Male C57/BL6 mice are injected with ENU (3 weekly injections of 85mg ENU/kg mouse) and

bred to a normal female C57/BL6 mouse to generate G1 offspring. Male G1s are then bred to normal female C57/BL6 mice to generate G2s. Female G2s are then backcrossed to G1 fathers to generate G3 mice which are phenotyped for homozygous recessive mutations. To identify immune mutants, mice at six to eight weeks of age are bled and lymphocyte populations are analyzed by flow cytometry.

Mice with a mutant phenotype thus identified are outcrossed to a different mouse strain which differs from C57BL/6 in multiple single nucleotide polymorphisms (SNPs) to generate F1s. For *Ms. T-less* and most immune mutants identified, we have chosen 129/SVImJ as the preferred mapping strain. F1s are intercrossed to generate F2s which are phenotyped and then subjected to SNP mapping to identify chromosomal regions that are homozygous C57BL/6J only in the affected animals. Concurrently, affected G3 animals are crossed to C57BL/6J to determine heritability of the mutation.

Example 2 Characterization of T cell Development in *Ms. T-less* Mutant Mice Lacking Functional IP3KB

This Example describes effects of the *Ms. T-less* mutation on T cell development and functions. *Ms. T-less* (family 7) is a mutant that exhibits very few T cells in the peripheral blood, based on results from analyses of CD3 expression in peripheral blood lymphocyte populations. It was found that peripheral blood CD4+ and CD8+ T cells, but not B cells, are drastically reduced in *Ms. T-less* mutant mice. As shown in Fig. 1, peripheral blood lymphocytes from mutant (squares on the left side of each panel) and control (wt, squares on the right side of each panel) mice on a C57BL/6J background were stained with antibodies to CD3, B220, CD4, and CD8. The Scatter plots show lymphocyte subpopulations as percentages of total lymphocytes. Mutants exhibit a profound reduction in T cell, but not B cell, percentages when compared to wildtype mice. Further, splenocytes from mutant and wildtype mice were stained with antibodies against CD4 and CD8 and analyzed by flow cytometry. As shown in Figure 2, spleens from *Ms. T-less* mutant mice are devoid of CD4+ and CD8+ T cells. *Ms. T-less* mice show drastically reduced T cell populations in the spleen. In older mice, however, there does seem to be a small accumulation of CD4+ cells. Numbers represent the percentage of cells within each quadrant.

In addition, T cells from mutant mice exhibit an activated phenotype.

Splenocytes from mutant and wt mice were isolated and stained with antibodies against CD4, CD8, CD44, and TCR β . The few CD4+ T cells seen in aged *Ms. T-less* mice do express TCR β . The results also indicate elevated expression of CD44. Expression of the TCR β chain and high levels of CD44 suggest that these cells may have expanded in a manner reminiscent of homeostatic proliferation to fill a lymphopenic environment.

As indicated in Fig. 3, the *Ms. T-less* mice exhibit a block in T cell development at the double positive (DP) stage. Thymi from mutant and wt mice were analyzed by flow cytometry using antibodies against CD4 and CD8. *Ms. T-less* mutant mice reveal an almost complete block at the CD4+CD8+ double positive (DP) to CD4+CD8- or CD4-CD8+ single positive (SP) transition. Numbers represent the percentage of cells in each quadrant.

Thymocytes from mutant and wt mice were analyzed by flow cytometry using antibodies against CD4, CD8, and TCR $\gamma\delta$. The results indicate that TCR $\gamma\delta$ T cell development in the *Ms. T-less* mutant appears to be normal. TCR $\gamma\delta$ T cells differentiate from TCR $\alpha\beta$ T cells prior to the DP stage. Therefore, the developmental block in *Ms. T-less* mice is specific for the TCR $\alpha\beta$ T cell compartment, likely resulting from a specific defect in thymic selection rather than a generalized impairment of T cell development.

It was found that DP thymocytes from *Ms. T-less* mice are not being selected. Expression analysis of activation markers and T cell receptor (TCR) associated genes suggests that the DP cells in the *Ms. T-less* mice are not receiving or improperly translating differentiation signals originating from the TCR. Thymi from mutant and wt mice were analyzed by flow cytometry using antibodies against CD4, CD8, CD69, and CD3 to follow T cell development. Shown in the figure are expression of CD69 and CD3 on CD4+CD8+ DP cells. The results indicate that DP cells from *Ms. T-less* mice are not being properly selected as evidenced by lack of expression of activation markers such as CD69 and a lack of CD3^{hi} cells.

In addition, the thymic cellularity of mutant and wt mice was determined by Trypan blue exclusion. Cell numbers were normalized to those observed in sex and age-matched wt littermate controls. Cellularity is expressed as a fold change value of the mutants over wt (for this analysis, n=8). The results indicate that the *Ms. T-less* mutant exhibits thymic hypercellularity despite a lack of positive selection.

Splenic B cell maturation appears altered in *Ms. T-less* mice. Splenocytes from mutant and wt mice were analyzed by flow cytometry using antibodies against IgM and IgD to follow B cell (B220+ cell) maturation. The results indicate that mutant mice have a possibly elevated representation of IgM⁺IgD^{dull/-} transitional type 1 (T1) cells, a decreased percentage of IgM⁺IgD⁺ transitional type 2 (T2) cells, yet a normal percentage of IgM^{dull}IgD⁺ mature cells.

Example 3 Normal Ca²⁺ Responses in *Ms. T-less* Thymocytes

ITPKB converts IP3 to IP4. IP3 is a well characterized second messenger involved in calcium signaling. TCR ligation leads to activation of phospholipase C γ (PLC γ), which hydrolyzes phosphatidylinositol (4,5) bisphosphate (PIP2) to diacylglycerol (DAG) and IP3. The augmentation of intracellular IP3 levels triggers the release of Ca²⁺ from internal stores via IP3 receptors. In Jurkat cells, ITPK activity and IP4 production are elevated upon TCR stimulation. Thus, ITPKB could serve to limit TCR induced Ca²⁺ mobilization via conversion of IP3 to IP4. However, it has also been postulated that IP4 can potentiate IP3 signaling via specific inhibition of a 5'-phosphatase that hydrolyzes IP3 to inositol (1,4) bisphosphate (IP2). Thus, formation of IP4 could also affect Ca²⁺ mobilization positively. We therefore investigated whether defects in Ca²⁺ signaling might underlie the developmental defect in *Ms. T-less* thymocytes.

The results indicate that DP thymocytes from *Ms. T-less* mice exhibit a normal calcium flux in response to CD3 crosslinking, as well as to ionomycin and thapsigargin stimulation. Thymocytes from mutant and wt mice labeled with Fura Red and Fluo-4 were either treated with biotinylated anti-CD3 antibody crosslinked with streptavidin, or stimulated with ionomycin or thapsigargin. The calcium flux of DP cells was determined as the ratio of the two calcium dyes measured over time after stimulation. The data suggest that *Ms. T-less* thymocytes have no major defects in internal Ca²⁺ release or external Ca²⁺ influx.

We next investigated the Ca²⁺ responses of individual cells to TCR stimulation. Thymocytes undergoing positive selection display dramatic oscillations of intracellular Ca²⁺ levels which have a periodicity on the order of seconds. Cells which do not receive signals for positive selection do not show the same magnitude of oscillations at the single cell level. In HeLa cells, ITPK activity and IP4 have been implicated in the

modulation of histamine-induced Ca^{2+} oscillations. Surprisingly, single cell imaging of Fura-2 labeled thymocytes did not reveal significant differences in magnitude or periodicity of TCR-induced Ca^{2+} oscillations between wt and mutant mice. Taken together, these data suggest that defects in Ca^{2+} signaling are unlikely to underlie the profound defect in T cell development observed in *Ms. T-less* mice.

Example 4 The Defect in T cell Development Is Inherent to the T cells

The DP block of T cell development in *Ms. T-less* mice is reminiscent of the phenotype seen in mice lacking both MHC I and MHC II proteins. However, we found no major differences in MHC protein expression between wt and mutant animals. This does not preclude the possibility that an unknown ligand which is necessary for differentiation into mature CD4^+ or CD8^+ T cells could be presented on the thymic epithelium and lacking in *Ms. T-less* mice. To address this issue, we performed bone marrow reconstitution experiments into lethally irradiated, B6.SJL hosts. The irradiation depletes the hosts of their complement of hematopoietic cells and precursors, yet keeps the thymic epithelium intact. We observed a profound block of T cell development at the DP stage in hosts reconstituted with *Ms. T-less* bone marrow. In addition, no T cells were present in the periphery, although B cells reconstituted efficiently. Bone marrow from wt mice exhibited normal T cell development in the host thymus. Wild type bone marrow reconstitution into lethally irradiated *Ms. T-less* hosts exhibited normal T cell development in the thymus. These findings indicate that the developmental block in T cell maturation is intrinsic to the developing thymocytes and does not depend upon ligands on, or signals from the thymic epithelium.

Example 5 *Ms. T-less* Mutant Mice Harbor a Nonsense Mutation in the IP3KB Gene

To determine the genetic lesion underlying the *Ms. T-less* phenotype, mutant mice on a C57BL/6 background were crossed to wt 129SvJ mice. SNP genotyping of multiple phenotypically mutant or wt F2 offspring revealed a perfect phenotype-genotype correlation for a 2 MB interval distal on chromosome 1. Analysis of this region did not reveal any obvious candidate genes known to be involved in thymic development. Thus, we examined the expression status of most known or predicted genes in the region using the GNF Gene Expression Atlas (<http://expression.gnf.org>). We found that the *Itpkb* transcript

accumulates in both murine and human lymphoid tissues, especially the thymus.

Sequencing of this candidate gene revealed a T to A transversion at position 596 of the transcript, changing the codon encoding cysteine 199 to a stop. The mutant transcript encodes an N-terminally truncated ITPKB protein lacking most of its structure, including domains that are involved in targeting and regulation, as well as the catalytic domain.

Immunoblot analyses of lysates from sorted CD4⁺ CD8⁺ DP cells, or of immunoprecipitates from whole thymocyte extracts, revealed that *Ms. T-less* thymocytes lack full length ITPKB protein. Expression of *Itpkb* RNA, however, is quite abundant in sorted mutant DP thymocytes. In agreement with these data, extracts from *Ms. T-less* thymocytes showed an approximately 50% reduction of ITPK activity compared to wt extracts. This residual activity could reflect low level thymic expression of other ITPK isoforms. Our data suggest that lack of full length ITPKB protein expression and concomitant reduction of ITPK activity in thymocytes underlie the defect in T cell development in *Ms. T-less* mice.

Example 6 Defective Erk Activation in *Ms. T-less* Thymocytes

The lack of an overt effect on Ca²⁺ responses in *Ms. T-less* thymocytes led us to consider other mechanisms of how IP3KB could control T cell selection. Since IP3 levels do not seem to be affected in these mice, we addressed putative mechanisms involving IP4. The protein GAP1^{IP4BP} has been shown to bind IP4 with high affinity and specificity *in vitro*. GAP1^{IP4BP} is a GTPase activating protein (GAP) that stimulates the small GTPase Ras to convert GTP to GDP, rendering Ras inactive. Several studies have demonstrated essential roles for the Ras pathway in T cell development. It has been shown that functional inactivation of the Ras activator RasGRP, Ras, or components of the MAP-kinase pathway downstream of Ras, all affect maturation of DP thymocytes and positive selection. Therefore, GAP1^{IP4BP} could be an important component which connects IP3KB mediated IP4 production to Ras activation in T cell development.

To address activation of the Ras pathway in *Ms. T-less* thymocytes, cells were stimulated with either a suboptimal TCR signal using α CD3 alone or with a maximal TCR signal using a combination of α CD3 and α CD4 antibodies. Ras activation leads to the activation and phosphorylation of the MAP kinases Erk1 and Erk2. Via immunoblot

analysis, we found a significant impairment of Erk1 and Erk2 activation in response to suboptimal (α CD3 alone) stimulation in *Ms. T-less* thymocytes. Optimal stimulation conditions (α CD3 and α CD4) or stimulation with the diacylglycerol (DAG) analog phorbol 12-myristate 13-acetate (PMA), which can localize RasGRP1 to the plasma membrane to allow for Ras activation, elicited normal levels of Erk1 and Erk2 activation.

These data demonstrate that *Ms. T-less* thymocytes are unable to efficiently activate Erk1 and Erk2 under moderate stimulation conditions. Thus, the block during positive selection in *Ms. T-less* thymocytes may reflect critical roles for IP3KB and its product IP4 as regulators of TCR induced Ras activation.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

All publications, GenBank sequences, patents and patent applications cited herein are hereby expressly incorporated by reference in their entirety and for all purposes as if each is individually so denoted.